J Membrane Biol (2010) 236:163–166
DOI 10.1007/s00232-010-9288-8

# The Effect of High-Frequency Electric Pulses on Tumor Blood Flow In Vivo

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Received: 9 July 2010 / Accepted: 12 July 2010 / Published online: 28 July 2010


**Abstract** The aim of this study was to evaluate the effect of a 5-kHz repetition frequency of electroporating electric pulses in comparison to the standard 1-Hz frequency on blood flow of invasive ductal carcinoma tumors in Balb/C mice. Electroporation was performed by the delivery of eight electric pulses of 1,000 V $cm^{-1}$ and 100 μs duration at a repetition frequency of 1 Hz or 5 kHz. Blood flow changes in tumors were measured by laser Doppler flowmetry. Monitoring was performed continuously for 10 min before application of the electric pulses as well as immediately after application of the electric pulses for 40 min. The delivery of electric pulses to tumors induced changes in tumor blood flow. The reduction in blood flow started after the stimulation and continued for the 40-min period of observation. There was a significant difference in blood flow changes 3 min after application of the electric pulses at 1-Hz or 5-kHz repetition frequency. However, after 3 min the difference became nonsignificant. The findings showed that the high pulse frequency (5 kHz) had an effect comparable to the 1-Hz frequency on tumor blood flow except at very short times after pulse delivery, when pulses at 5 kHz produced a more intense reduction of blood flow.

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**Keywords** Electroporation · Electropermeabilization · Invasive ductal carcinoma · Laser Doppler flowmetry · Blood flow

## Introduction

Cell membrane electropermeabilization is a physical method that uses short and intense electric pulses to increase membrane permeability (Neumann et al. 1982) and therefore increases the uptake of molecules such as DNA, antibodies and drugs into the cells (Mir 2000). Electrochemotherapy (ECT) is one application in which the toxicity of a drug may locally be increased by increasing the permeability of the target cells (Mir et al. 1991). Several studies have provided evidence that ECT is an effective treatment for local tumors in patients with different cancer types (Marty et al. 2006; Sersa et al. 2008a). Experiments have provided sufficient data to demonstrate that ECT with either bleomycin or cisplatin is highly effective in the treatment of solid tumors.

In spite of its advantages, there are also some side effects with ECT (Sersa et al. 2008a). In ECT, the pulse delivery is usually painful for patients and causes a muscle contraction. Several electric pulses are usually delivered, with a repetition frequency of 1 Hz, which results in several individual sensations and muscle contractions (Zupanic et al. 2007).

To reduce the number of individual muscle contractions, use of a pulse frequency larger than the frequency of tetanic contraction has been suggested (Miklavcic et al. 2005). It was reported that a train of eight electric pulses at 1 Hz results in eight muscle contractions, while the same number of pulses results in only one single muscle contraction when pulses are delivered at 5 kHz. Increasing the

repetition frequency of the pulses lowers the number of contractions, whereas clinical effectiveness remains at the same level as that of the pulses delivered at 1 Hz (Marty et al. 2006). This means that high electric pulse repetition frequency not only shortens the therapy session but also allows the ECT to be better tolerated by the patient in comparison to the standard pulses at 1 Hz (Zupanic et al. 2007).

It has been shown that the antitumor effectiveness of ECT is mainly related to increased drug uptake caused by electroporation (Poddevin et al. 1991; Sersa et al. 2006; Kanthou et al. 2006). In vivo, electrical pulses have other effects, such as a transient and reversible reduction of tissue blood flow (Sersa et al. 1998, 1999, 2006; Gehl et al. 1999, 2002; Kanthou et al. 2006). Reduction of tumor blood flow may result in trapping of the drug in the tumors, thus providing a longer time for the drug to act (Mir 2006). Modification of the blood flow could be particularly beneficial for intratumoral drug administration as this would decrease the drug washout from the tumor (Mir 2006).

In vitro studies have reported that with an increased repetition frequency (up to 8.3 kHz) uptake of nonpermanent molecules might stay at similar levels, but the field strength necessary to achieve optimal uptake had to be increased (Pucihar et al. 2002). However, under usual conditions, ECT with high repetition frequencies inhibited tumor growth and had similar treatment efficiency regardless of the pulse frequency used, 1 Hz or 5 kHz, in vivo in mice. It was demonstrated that increasing the repetition frequency of the electric pulses from the standard 1 Hz to 5 kHz was also successful in human clinical cases (Miklavcic et al. 2005; Marty et al. 2006; Snoj et al. 2007). Gehl et al. (2002) analyzed the influence of the voltage and the duration of the pulses on the vascular lock. To the best of our knowledge, there has been no experiment on the tumor perfusion changes as a function of the repetition frequency of the electric pulses.

The purpose of present study was to investigate the changes in tumor perfusion after application of electric pulses at 1-Hz and 5-kHz repetition frequencies.

## Materials and Methods

### Mice and Tumors

Healthy female Balb/C mice, 6–8 weeks of age, were purchased from the Pasteur Institute (Tehran, Iran). They were kept at 22°C temperature with a natural day/night cycle for 10 days for adaptation. Spontaneous mouse mammary tumor (SMMT), i.e., an invasive ductal carcinoma, was transplanted by implanting a 4-$mm^3$ fragment into the right flank of anesthetized mice. Approximately 2 weeks after tumor transplantation, when the largest tumor diameter exceeded 5 mm (measured by caliper), the animals were randomly divided into experimental and control groups (15 animals for the control group and for each of the experimental groups).

### Electroporation Protocol

Two flat, parallel, stainless-steel plate electrodes were placed at the opposite sides of the tumors to deliver the electric pulses. Conductive gel was used to assure good contact between electrodes and skin. Eight square-wave electric pulses of 1,000 V $cm^{-1}$, 100 μs duration, at repetition frequencies of 1 Hz or 5 kHz, were delivered to the mice using an ECT-SBDC device for electroporation that was designed in the Electromagnetics Laboratory of the Medical Physics Department of Tarbiat Modares University (Tehran, Iran).

### Laser Doppler Flowmetry

Relative blood flow of tumors was measured using laser-Doppler flowmetry (MBF3; Moor Instrument, Axminster, UK) by inserting the probes (MP3/MP3b, hypodermic stainless-steel tube) into the tumor via a small superficial incision on the skin (Jarm et al. 2002). Laser-Doppler flowmetry is widely used for the assessment of microvascular blood perfusion in tissue, and the operating principles are described, e.g., in Shepherd and Oberg (1989). The measured microvascular blood flow was the product of the mean red blood cell velocity and the mean red blood cell concentration in the volume of tissue being monitored. Microvascular blood flow is expressed in relative units called "blood perfusion units."

The output of the electroporation device was connected to a personal computer via an RS232 connector. Microvascular blood flow signals were stored on the computer at a frequency sample rate of 40 Hz. Mean flow values over 5 min (Fig. 1) or 1 min (Fig. 2) were calculated using the Winsoft program (laser Doppler perfusion measure, V3.0.8; Moor Instruments).

All experimental measurements were conducted on anesthetized mice to minimize discomfort and movements. Mice were anesthetized by means of intraperitoneal administration of 0.01 ml/g body weight of the following mixture: 0.5 ml of ketamine (100 mg/ml; Virbac, Carros, France), 0.5 ml of xylazine (2%; Bayer HealthCare, Leverkusen, Germany) and 4 ml of NaCl.

Anesthetized mice were placed on a heating pad to maintain the body temperature at a constant level. Rectal and surface temperature probes were used to control the temperature during the experiments. Rectal temperatures were kept at 37 ± 0.5°C by adjusting the heating pad

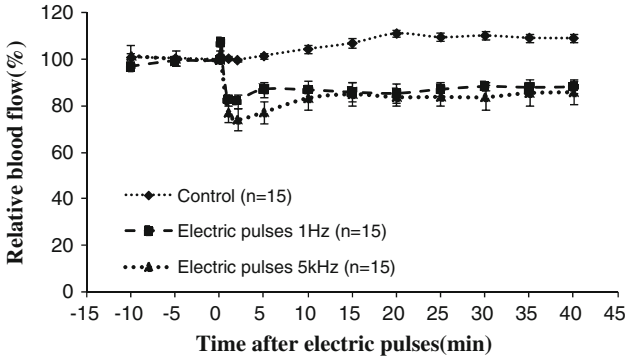


**Fig. 1** Blood flow changes (mean flow values over 5 min) at 5-min time intervals for 40 min following application of electric pulses at different frequencies

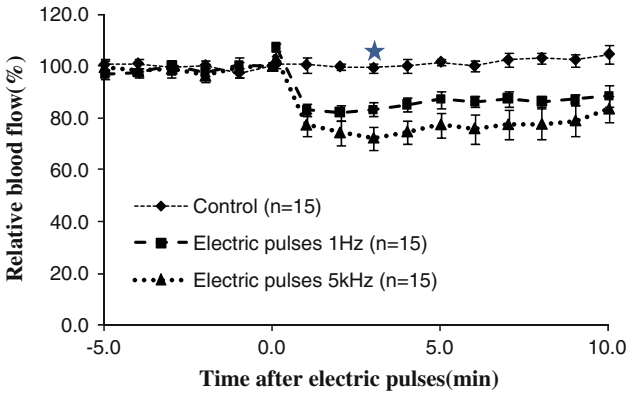


**Fig. 2** Blood flow changes (mean flow values over 1 min) during the initial period (10 min) after the pulses (* $P < 0.05$)

temperature. The ambient temperature was 22–24°C during the experiments.

Approximately 5 min after anesthesia, the laser-Doppler flowmetry probe was inserted into the tumor after making a small superficial incision in the skin. Data were acquired while the probe was strengthened in the tissue. After initial rest and stabilization (15 min), baseline recordings were performed for 10 min. Then, the electrodes were placed as described above and the electric pulses delivered to the tumors. The flowmetric recording was continued after the pulses for at least 40 min. In the control group, the same procedure was performed; however, no electric pulse was delivered. Special care was taken during the measurements to minimize the movements of probes and mice during acquisition. Average blood flow values over 5 min before electric pulse delivery and at 0, 5, 10, 15, 20, 25, 30, 35 and 40 min after the electric pulses were calculated. Blood flow changes after the pulses were normalized with respect to the average blood flow obtained during the 10 min before time zero. Thus, blood flows were expressed as percentages of the preexposure blood flow.

### Data Analysis

The average perfusion changes due to treatment versus the electrical pulse duration were plotted for each group of mice. All data were tested for normality of distribution. ANOVA and unpaired Student's $t$ test were used to evaluate the statistical significance of differences between the experimental and control groups. $P < 0.05$ was considered significant in the statistical tests.

## Results

In this study, laser-Doppler flowmetry was used to evaluate the blood flow variations induced by application of electric pulses to tumors in vivo. Tumor blood flow was measured continuously from 10 min before application of the electric pulses up to 40 min after the electric pulses. In this way, we were able to monitor even short-lived and/or small changes in blood flow.

Eight pulses of 100 μs duration were delivered at a repetition frequency of 1 Hz or 5 kHz. The repetition frequency of the pulses was changed through adjustment of the delay between two consecutive pulses in the train.

The changes in tumor blood flow as a function of the frequency repetition are shown in Fig. 1. The curves in Fig. 1 represent the blood flow changes in control and in the groups treated at 1 Hz and 5 kHz and monitored for 40 min after pulse delivery.

There was a rapid reduction in blood flow immediately after application of the pulses. Minimal blood flow level was reached at 2 min after pulse delivery. The rapid reduction was followed by a gradual reperfusion. The partial reperfusion reached a peak around 10–20 min. There was a difference between the changes in tumor blood flow after application of electric pulses at 1 Hz or 5 kHz. At the repetition frequency of 5 kHz, the reduction in tumor perfusion was higher than at the frequency of 1 Hz (Fig. 2). The difference was significant at 3 min after pulse delivery, and the difference in the perfusion between the two repetition frequencies completely disappeared after 10 min, remaining similarly lower with respect to the controls. The tumor blood flow in the control group showed no significant changes during the same monitoring period.

Results of the $t$ test revealed significant differences in blood flow between control and each experimental group immediately after application of electric pulses ($P < 0.05$). These differences remained significant until the end of the monitoring, 40 min after application of the pulses. The difference between the groups treated with the electric pulses at 1 Hz and 5 kHz was significant ($P < 0.05$) only at 3 min after electric pulse delivery.

## Discussion

ECT is an efficient local treatment of cutaneous and subcutaneous tumors. However, muscle contractions and painful sensations are unpleasant effects during electric pulse delivery (Sersa et al. 2008a). To reduce the painful sensations of the treatment, pulses delivered at a repetition frequency of 1 Hz may be replaced by pulses delivered at 5 kHz, while preserving the efficiency of the treatment (Miklavcic et al. 2005).

It is now a well-documented fact that electroporation has a vascular disrupting action (Gehl et al. 2002; Kanthou et al. 2006). Electroporation-based therapies cause changes in blood flow and vascular permeability (Gehl et al. 2002; Sersa et al. 2008b). The duration of the vascular lock consequently starves the tumor of oxygen and nutrients and contributes to the antitumor effects of ECT.

Although the vascular effects of electroporation have been shown by different techniques (Sersa et al. 2008a), they have not yet been monitored in real time quantitatively. Therefore, it seemed useful to evaluate the effect of electric pulses of different frequencies on blood flow of tumors in real time. In this study, the effects of the 1-Hz and 5-kHz pulse frequencies were investigated on the blood flow of a mouse tumor model.

Our study demonstrates that application of electric pulses induces significant changes in tumor blood flow (Fig. 1). Our results were consistent with those of previous investigations that reported a reduction in blood flow after application of electric pulses (Sersa et al. 1999). We observed an immediate reduction in blood flow after application of electric pulses that gradually (but incompletely) recovered for the 40 min monitoring period. The immediate reduction in tumor blood flow at 5 kHz was higher than the reduction at 1 Hz during the initial period following pulse delivery. Our data also showed that at longer times the higher pulse frequency (5 kHz) had effects on tumor blood flow comparable to those observed at 1 Hz.

Our results are thus in agreement with those of clinical studies showing no difference in the effectiveness of ECT using repetition frequencies of the applied pulses of 1 Hz or 5 kHz (Marty et al. 2006; Snoj et al. 2007).

**Acknowledgements** E. R. wishes to especially thank Prof. Lluis Mir (Laboratoire de Vectorologie et Thérapeutiques Anticancéreuses, UMR 8203 CNRS Université Paris-Sud, Institut Gustave-Roussy, Villejuif, France), without whom the article would not have reached a satisfactory level of presentation.

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